DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

Version
January 1997

Compiled by

THE DIVISION OF AIDS

NATIONAL INSTITUTE OF ALLERGY & INFECTIOUS DISEASES

NATIONAL INSTITUTES OF HEALTH

and

COLLABORATING INVESTIGATORS

HIV PROVIRAL POLYMERASE CHAIN REACTION (HIV DNA PCR)

I. PRINCIPLE

HIV infection is usually documented by detection of HIV specific antibodies in serum. However, serologic assays do not readily identify HIV infection in neonates with passively acquired maternal antibodies or individuals with "indeterminate" antibody profiles or early infection. These situations have often required follow-up serologic testing, antigen detection and culture.

Gene amplification techniques, e.g., Polymerase Chain Reaction (PCR), allow the detection of rare DNA sequences. PCR can detect low copy numbers (1-5) of HIV-1 proviral DNA in infected cells. PCR may help document HIV infection when routine diagnostic assays are not adequate.

Characteristic of all retrovirus infections, the HIV single stranded RNA is transcribed in double stranded DNA ("provirus") and integrated into the host cell genome. This integrated HIV provirus can be detected by amplification of a specific sequence in the highly conserved gag region of the genome.

Whole blood is treated with a specimen wash reagent which selectively lyses the red blood cells. The intact leukocytes are pelleted and washed. Alternatively, the pellets can be made from the PBMCs isolated using lymphocyte separation techniques described in the Qualitative PBMC Macrococulture Assay Protocol. The pellets can be stored at -70^0C or be treated with nonionic detergents, magnesium and proteinase K to extract the DNA for analysis.

The double stranded DNA is denatured by heat to expose the target region to the labeled primers. The amplification target is a highly conserved region of the gag genome which is bordered by the primer pair SK 431/462. Two biotinylated oligonucleotide primers complementary to the amplification target sequence will bind to the target region on the DNA. The taq polymerase utilizing deoxynucleotide triphosphates, (dATP, dGTP, dCTP, dUTP) extend in the 5' to 3' direction to produce biotinylated complementary DNA sequence called amplicons. During the polymerase chain reaction, controlled fluctuations in temperature allow repeated denaturation, annealing and extension processes resulting in a geometric increase in the target sequences. DNA copies from previous cycles become templates in subsequent amplification periods.

Uracil-N-Glycosylase (UNG) is used to prevent contamination from previous PCR reactions. UNG utilizing strand excision, will excise any dUTP found in previously amplified DNA. Naturally occurring DNA will contain dTTP. UNG is only active at 55^0C. Before the PCR reaction is initiated, the thermocycler is set at 55^0C to activate UNG for two minutes. After the amplification cycles are completed, the UNG is inactivated by a denaturing solution containing sodium hydroxide.

The amplified DNA is incubated onto polystyrene wells which contain immobilized BSA-conjugated probe (SK 102) which is specific to the biotinylated amplicons. After incubating for one hour, the unbound reactants are washed away. An avidin-horseradish peroxidase conjugate is added and incubated for 15 minutes at 37 $\pm$ 1^0C. Unbound reactants are washed away. A chromogentic substrate, tetramethylbenzidine (TMB) is added for a 10 minute incubation. The reaction is stopped by the addition of H_2SO_4 and the absorbance is read at 450 nm. A fixed O.D. cutoff of 0.350 is used to determine whether a specimen is positive or negative.

II. SPECIMEN REQUIREMENTS

Acceptable specimens include whole blood collected in Acid Citrate Dextrose (yellow top) vacutainer or in EDTA (lavender top) vacutainer. Heparinized (green top) blood is not acceptable for this assay. Technically other body fluids may be processed for HIV PCR. However, the HIV PCR assay has been standardized using peripheral blood specimens.

Specimens should be transported and stored at 2-25^0C. Whole blood should be processed to PBMC pellets within 4 days of collection. Specimens should not be frozen prior to separation of PBMCs.

Ill. HIV PCR REAGENTS: Roche - Amplicor Detect™ kits, listed in A, B and C below:

A. Whole Blood Specimen Kit:

1. Specimen Wash Solution (H_2O, Sodium Chloride, Saponin)
2. Extraction reagent (Tween 20, H_2O)

B. HIV-1 Amplification Kit:

1. HIV-1 Positive Control
2. HIV-1 Negative Control
3. H IV-1 Master Mix
4. HIV-1 AmpErase

Positive control: 50 µL positive control + 200 µL extraction buffer
Negative control: 50 µL negative control + 200 µL extraction buffer

C. HIV-1 Detection Kit:

1. Wash Concentrate (H_2O, Sodium Chloride, Tween 20)
2. PCR Denaturation Solution (Sodium Hydroxide, EDTA, H_2O)
3. PCR Hybridization Buffer (Sodium Thiocyanate, Sodium Phosphate, H_2O)
4. PCR Conjugate (TRIS, Avidin-HRPO)
5. PCR Substrate A (H_2O_2, Citric Acid, H_2O)
6. PCR Substrate B (DMF, H_2O, TMB)
7. Stop Reagent (Sulfuric Acid, H_2O)

D. Standards and controls provided by the Virology Quality Assurance Laboratory (VQA)
   1. 0 copy standard
   2. 5 copy standard
   3. 10 copy standard
   4. 20 copy standard
   5. Blinded QC samples

IV. SUPPLIES AND EQUIPMENT

A. Sample Processing / Extraction Room:

40-200 µL pipettor
200-1000 µL pipettor (2)
2 mL sterile Sarstedt tubes
3 mL sterile plastic disposable transfer pipettes
12 x 4 Sarstedt tube rack
2 heating blocks
Table top microcentrifuge

B. Amplification Reaction Room:

0.5-10 µL pipettor
10-50 µL pipettor
40-200 µL pipettor
200-1000 µL pipettor
Eppendorf repeat pipettor
Retainer tray & holder
Aerosol resistant pipettor tips
500 µL snap cap microfuge tubes
1.5 mL snap cap microfuge tubes
1.25 mL sterile Eppendorf repeater tips
Thin-walled reaction tubes & caps

C. Product Room:

0.5-10 µL pipettor
10-50 µL pipettor
40-200 µL pipettor
200-1000 µL pipettor
Multichannel pipettor (5-50 µL)
Multichannel pipettor (50-200 µL)
Aerosol resistant pipettor tips
Retainer tray holder
Retainer tray holder

10 mL pipettes
Sterile reagent boats
Table top microfuge
DNA thermal cycler Perkin-Elmer TC 9600 (Note: if using a different thermocycler adjust program instructions listed below to define appropriate cycle parameters.)

V. PROCEDURE:

Call up an assay template and match specimens with the list. Be sure specimens were stored appropriately, i.e., at 2-8^{0}C.

A. Sample Processing:

Protective Clothing: Disposable isolation gown, shoe covers, face mask, 2 disposable gloves
Location: Sample Preparation Area with a P2 Biological Safety Cabinet

1. For each patient specimen, pipette 1.0 mL specimen wash buffer into a 2.0 mL Sarstedt vial. Note: Each patient sample is run in duplicate.

2. Invert blood specimen in vacutainer tube 15-20 times to mix thoroughly. Add 0.5 mL whole blood to vial containing specimen wash buffer.

3. Seal vial and mix by inversion 10- 15 times.

4. Allow mixture to stand at room temperature for 5 minutes. Vortex specimen vial thoroughly for a minimum of 15-30 seconds. Incubate specimen at room temperature for 5 minutes. Vortex specimen thoroughly for a minimum of 15 - 30 seconds.

5. Centrifuge vial for 3 minutes at 1200 rpm at room temperature in a table top microfuge.

6. Aspirate supernatant being careful to avoid disturbing the pellet. Add 1.0 mL specimen wash buffer to vial. Vortex vial specimen thoroughly for 30 seconds. Centrifuge vial for 3 minutes at 1200 rpm at room temperature.

7. Repeat Step 6 (2nd wash).

8. Aspirate supernatant being careful to avoid disturbing the pellet. The dry pellet may be extracted immediately or stored at -70^{0}C until ready to extract.

NOTE: Pellets may also be prepared from the PBMCs isolated using the lymphocyte separation techniques described in the Qualitative PBMC Macrococulture Assay.

B. Extraction:

Protective Clothing: Disposable isolation gown, shoe covers, face mask, 2 disposable gloves.

Location: Set-Up Area

Check that the 2 separate heating blocks are heated to 60^0C and 100^0C.

1. For each run include a minimum of 3 kit negative controls and 1 kit positive control. Include also one pellet each of VQA 0 Copy, 5 Copy, 10 Copy, 20 Copy Standards and two pellets of Blinded QC Samples.

2. Remove cell pellet from -70^0C. To each pellet, add 200 µL of extraction reagent, vortex for 15-30 seconds. Incubate at 60^0C for 30 minutes.

3. Immediately incubate extract at 100^0C for 30 minutes. Proceed to PCR amplification procedure.

C. Amplification Reactions:

Protective Clothing: Disposable isolation gown, shoe covers, face mask, 2 disposable gloves

<u>Reconstitution of 2x Master Mix-Amperase</u>

Location: "Clean" Pre-PCR "No DNA" BSC Area

1. Generate a plate map from the laboratory computer. Intersperse the Kit Negative controls throughout the patient samples and controls.

2. Add 100 µL of Amperase into one vial of Master Mix. Invert mixture 10-15 times. This mixture is sufficient for 32 amplifications. Write the date of the Master Mix-Amperase preparation onto the vial. This mixture is stable for 4 weeks at $2\text{-}8^0$C.

3. Place the PCR tubes into the sample retainer tray. Aliquot 50 µL of the 2x master mix into each PCR tube.

Location: "Clean" Pre-PCR "DNA OK" Area

4. Aliquot 50 µL of the extracted sample DNA into the master mix of the appropriate tube. Use positive displacement pipettors and a separate sterile ART pipette tips for each specimen. <u>Use extreme care to avoid carry-over contamination and aerosols</u>. Loosely cap each strip of PCR tubes after inoculation. After all tubes have been inoculated, firmly press caps on using the installation tool.

5. Remove tubes from the retainer tray base. Carry PCR tubes in the white retainer tray directly into the product room. Place retainer tray into the TC 9600 thermal cycler, aligning the notch on the retainer tray with the notch on the TC 9600. Close the cover and turn the cover knob until the white portions of the lid & knob are aligned.

6. a. Begin the HIV-1 proviral PCR program using the following file programs:

Hold:	50^0C,	2 minutes	
Cycle:	95^0C,	10 seconds	
	55^0C,	10 seconds	5 cycles
	72^0C,	10 seconds	
Cycle:	90^0C,	10 seconds	
	60^0C,	10 seconds	30 cycles
	72^0C,	10 seconds	
Hold:	72^0C,	5 minutes	
Hold:	72^0C	"forever"	

The HIV-1 DNA program series requires approximately 75 minutes to complete.

b. To start TC 9600 thermal cycler program:
   1) Turn on switch for TC 9600
   2) "Run" is underlined. Press: enter
   3) Screen: Run Enter Program #
   4) Screen: Reaction volume: "100?"
      Press: enter
   5). Thermal cycler begins. Program file runs for 75 minutes.

(Note: if using a different thermocycler, adjust program instructions listed below to define appropriate cycle parameters as listed above.)

7. As soon as cycles end, press "Stop". TC 9600 temperature ramps down to 25^0C. Allow the TC9600 to hold at 25^0C for 5 minutes to allow condensation to settle.

8. Remove caps very carefully to avoid aerosols. Immediately add 100 µL amplicon denaturation solution to each sample.

9. Let denatured amplicons stand at room temperature for 10 minutes before proceeding to microtiter plate hybridization assay.

D. Detection

Protective Clothing: Disposable isolation gown, shoe covers, 2 pairs disposable gloves.
Location: PCR Product room

MICROTITER PLATE HYBRIDIZATION ASSAY FOR THE DETECTION OF PCR AMPLIFIED HIV-1 SEQUENCES

1. Allow all the reagents to come to room temperature. Remove the appropriate number of microtiter strips from the foil pack and place onto the plate holder. Add 100 µL of hybridization/neutralization buffer to each well.

2. Add 25 µL denatured amplicon to wells containing neutralization/hybridization buffer. Tap plate gently 10-20 times to sufficiently neutralize the amplicons. Neutralization will be evident when the color changes from blue to yellow.

3. Cover microtiter plate with plate cover and incubate for 1 hour at 37^0C.

4. Prepare Working Wash Solution by adding 1 volume of wash concentrate (10x) to 9 volumes of distilled or deionized water. Mix well. After the incubation, wash plate 5 times manually or by using a microwell plate washer.

    a. For Manual washing:

        1) Empty contents of plate and tap dry on paper towels
        2) Pipette working wash solution to fill each well to top (400-450 µL). Let soak for 30 seconds. Empty out contents and tap dry.
        3) Repeat step 2) four additional times.

    b. For Automated washing, program washer to:

        1) Aspirate contents of wells
        2) Fill each well to top with working wash solution (~350-450 µL dependent on plate washer), soak for 30 seconds, then aspirate dry.
        3) Repeat step 2) four additional times. Tap the plate dry.

5. Add 100 µL of Avidin-HRPO conjugate into each well. Cover plate with plate cover and incubate at 37^0C for 15 minutes.

6. Wash microtiter plate as described in step 4.

7. Prepare Working Substrate reagent just prior to use:

    Mix 4 parts PCR chromagen reagent A
    +
    1 part PCR substrate reagent B

Add 100 µL Working Substrate reagent into each well and incubate exactly 10 minutes at room temperature in the dark.

8. Stop reaction with 100 µL stop reagent.

9. Read microtiter plate at 450 nm.

VI. QUALITY CONTROL

The controls must react as expected:

- Kit Positive control should have the A_{450} reading 2.0 or greater (preferably 3.0).
- Kit Negative control must be negative. A_{450} reading should be 0.250 or less.
- VQA 5,10, and 20 copy standards must be positive.
- VQA 0 copy standard must be negative.
- Blinded pellets will be evaluated by the VQA when the data is exported.

A run with one or more controls out of line will usually need to be repeated.

VI. REPORTING RESULTS

1. Absorbances 0.350 or greater are considered positive.

2. Absorbances below 0.350 are considered negative.

All specimens are run in duplicate. If these duplicates have discordant results, the assay will need to be reviewed and the specimens may require retesting.

VII. PROCEDURE NOTES

1. To avoid contamination of specimens and reagents:

   a. No aliquot is ever returned to the original container.

   b Only one tube or container is opened at a time.

   c. Use aerosol resistant tips for all pipettors.

   d. Use different tips or pipettes for each reagent or specimen.

   e. Do not insert pipettor beyond the disposable tip into any container.

2. The submitting physician or laboratory must be called if a specimen is deemed incorrect, inadequate or if there is a failed run.

3. At each step, specimen labeling and handling must be optimized to prevent mix up or contamination.

If any negative controls are positive, suspect contamination. Clean up the area and equipment with a solution of 1M NaOH or 1M HCl to remove any contamination with PCR product. Repeat the assay to verify results.

IX. REFERENCES

Butcher A, Spadoro J: Using PCR for detection of HIV-1 Infection. Clin Imm Newsletter 12:73:76, 1992.

Cushwa WT, Medrano SF: Effects of Blood Storage Time and Temperature on DNA Yield and Biotechniques 14:204-207, 1993.

Roche HIV PCR kit publications, 1992.